Study of Secondary Metabolites in *Piper chaba* Hunt. – A Medicinally Important Plant Species

Synopsis
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INTRODUCTION

Plants have been used to treat diseases, enhance wound healing, and reduce pain since the beginning of human civilization. Traditional medicine is an essential component of the primary health care system. Medicinal herbs played an active part, either as an active principal or as a conventional formulation (Kumar and Van Staden, 2016). Around 80% of people in developing nations use traditional medicines including medicinal plant-based compounds (WHO, 2008). The chemical components present in them are part of living flora's physiological functions and are therefore, believed to have better compatibility with the human body. Significance of plant-based medicines has grown all over the world as nearly 50% of medicines in the market are made from natural basic materials.

India comprises a wide range of medicinal plants and a rich floristic variety. It has a large range of tropical and agro-climatic conditions that are conducive to the introduction and domestication of new and exotic varieties of medicinal plants. More than 43% of the total flowering plants in India are stated to be medicinally essential (Pushpangadan, 1995). The annual demand for raw botanical drugs is very high in India's domestic market (Ved and Goraya, 2007). The rising demand for medicinal plants puts a significant strain on India's existing resources (Dhawan, 1997; Hazlett and Sawyer, 1998). Estimates show that more than 90% of the raw material is drawn from wild forms for pharmaceutical use (Uniyal *et al*., 2002).

Among the numerous medicinal plants, the genus *Piper* is a source of many medicinally important metabolites and essential oils (family: Piperaceae) and plays a significant role in the health of humans (Flores *et al.,* 2009). It comprises of 1000-2000 species of shrubs, herbs and lianas and most of them have oil cells containing important natural compounds. Bioactive compounds from *Piper* species have played a significant role as therapeutic agents in addition to their well-known usage as culinary spices (Barh *et al.,* 2013). Several species of *Piper* as. *P. nigrum, P. betle, P. longum, P. chaba* etc. are used in indigenous medicine systems (Parthasarathy *et al.,* 2006).

Piper chaba Hunt. is a flowering vine, native to South and Southeast Asia and commonly called Choi Jhal in Bangladesh and badi pippali in India (Ruangnoo *et al.,* 2012). The plant is creeper with 2 to 3 inches long oval-shaped leaves and, flowers are monoecious and bloom during the monsoon season. Fruits are elongated and up to 3 inches long. The fruiting spikes of *P. chaba* are known as “Java long

pepper". In Thailand, *P. chaba* is called 'Dee Plee". The stem, root, and fruit of *P. chaba* are commonly used in the recipe of traditional medicine (Islam *et al.,* 2020).

The importance of medicinal plant is due to the presence of secondary metabolites. Various significant bioactive compound identified in *P. chaba* are alkaloids Piperine, Piperanine, Pipernonaline, piperamine 2, 4-decadienoic acid piperidide, kusunokinin and pellitorine (Connolly *et al.,* 1995) Dehydropipernonaline, Piperlonguminine, Guineensine, Lignan (Bhandari *et al.,* 1998) are found in stem bark of the plant. A unique piperine dimer chabamide has been found in stem bark (Rukachaisirikul *et al.,* 2002). Piperine, Piplartine, Piperlonguminine, Sylvatine and β-sitosterol, N-isobutyl-(2E, 4E)-octadecadienamide, N-isobutyl-(2 E, 4 E, 14 Z)-eicosatrienamide, Retrofractamide B are some alkamides present in the root. The fruit oil of *P. chaba* contains caryophyllene oxide, β-caryophyllene, few monoterpenes hydrocarbons, aliphatic hydrocarbons and moderate amount of sesquiterpenes (Tewtrakul *et al.,* 2000).

P. chaba is a pungent aromatic stimulant and has anthelmintic and carminative properties also used to cure coughs as an expectorant, cold, bronchitis, piles, pain in the abdomen. It also possesses cholesterol lowering properties. Stem is useful in diarrhea, arthritis and rheumatic pains (Yusuf *et al.,* 1994). Chabamide, exhibited antimalarial, antituberculosis, and cytotoxic activities (Ren *et al*., 2015). Piperine, a pungent alkaloid and is the main compound present in *P. chaba* and has numerous important pharmacological activities, including antimicrobial, immunomodulatory, hepatoprotective, antioxidant, antimetastatic, and antitumor, among others (Stojanovíc-Radíc *et al.,* 2019). Other important pharmacological effects of piplartine include cytotoxic, genotoxic, antitumor, antiangiogenic, antimetastatic, antiplatelet aggregation, antinociceptive, anxiolytic, antidepressant, anti-atherosclerotic, antidiabetic, antibacterial, antifungal, leishmanicidal, trypanocidal, and schistosomicidal activities (Bezerra *et al.,* 2013).

Secondary metabolites, also known as natural products or phytochemicals, are unique to certain taxonomic groups and play a key role in interactions between plants and the environment, helping plants to protect themselves against pathogens or herbivores (Dixon, 2001; Kennedy and Wightman, 2011; Kliebenstein and Osbourn, 2012). Most plant secondary metabolites are very important for the everyday life of humans as sources of medicines. Plant secondary metabolites, previously unknown for

their pharmacological effects, have been extensively examined as a source of therapeutic entities in recent years (Krishnaraju et al.,2005). As a result, phytochemicals with adequate antimicrobial effectiveness are expected to be used in the treatment of microbial ailments (Balandrin et al.,1985).

Piperine is a cinnamoyl amide derivative. L-lysine is the source of the piperidine heterocycle of piperine (Leistner and Spenser, 1973), while the aromatic component of piperine is likely to be derived from the metabolism of phenylpropanoids. The aromatic part of the piperine is linked by an amide bond to a nitrogen-containing heterocycle, piperidine. For the decisive piperine synthase action, piperoyl-CoA is required, as reported by Geisler and Gross (1990). Information on the biosynthesis of secondary metabolites and the amides in the plant *P. chaba* is poor. Functional genomics is now routinely used for gene discovery in plant secondary metabolism, (Dixon, 2001). The implementation of Next Generation Sequencing (NGS) based RNA-sequencing technology has changed view on the transcription and regulation of the genome (Zhi Cheng *et al.,* 2013).

RNA sequencing (RNA-Seq) technology has been used to obtain full-scale transcriptomic information from various plant species and to provide deeper understanding into the transcriptional and post-transcriptional regulation of important genes in biosynthetic secondary metabolite pathways (Kalra *et al.,* 2013; Devi *et al.,* 2016). Genome sequencing using NGS could be useful for generating large molecular for identifying proteins (Yu *et al.,* 2011) involved in piperine biosynthesis.

Micropropagation is now extensively used to grow larger quantities of economically valuable crops in a widespread manner. This technique also can also be used as a method to increase the availability of the *P. longum* planting material. De novo shoot regeneration from callus cultures through organogenesis (Bhat *et al.,* 1992), direct shoot regeneration from leaf bases (Soniya and Das, 2002) and regeneration from shoot tips, root, leaf, node, internode, and petiole (Rani and Dantu, 2012) have been reported earlier. Rani and Dantu (2016) reported for the first-time *in vitro* shoot multiplication and mitigated browning in *Piper chaba* Hunt by using activated charcoal at the concentration 5mg/L as the most effective anti-browning agent.

Mintoo and Dantu (2019) reported for the first time the successful callusing, total antioxidant, total phenolics and flavonoids from the callus culture of *P. longum*; presence of piperine in callus and female

leaves at much lower level than spike and investigated the endophytic bacterial population, six endophytes were isolated and characterized belonging to ɣ Proteobacteria and Firmicutes group.

Furthermore, the study of secondary metabolites and their antimicrobial properties, as well as transcriptomics will reveal the rich genetic diversity associated with this plant, which is also expected to have agriculturally important properties.

OBJECTIVES

In this study, an attempt has been made to analysis the secondary metabolites, their antimicrobial properties and transcriptomics of *P. chaba*. Considering the importance of the plant species, the current study has been undertaken with the following objectives:

1. Establishment of *P. chaba* plants in DEI botanical garden.
2. Quantification of secondary metabolites in different parts of the plant.
3. Analysis of anti-microbial activity of secondary metabolites.
4. Transcriptomic analysis of different parts of the plant.

Review of Literature

Piperine is the primary bioactive substance of *Piper chaba* and *Piper longum*. Several studies have been conducted on the effects of piperine in terms of immunomodulatory, anti-carcinogenic, anti-asthmatic, stimulatory, hepatoprotective, anti-inflammatory (Darshan and Doreswamy, 2004), as well as antimicrobial, and anti-ulcer actions (Bai and Xu, 2000). It also has antioxidant properties and bio-transformation properties, and studies have shown that it increases the absorption of certain medicines, including phenytoin, rifampicin, sulphadiazine, and tetracycline.

The presence of piperine has been reported in roots and spikes of *P. nigrum* and *P. longum* (Upadhyay *et al.*, 2013; Chitlange *et al.*, 2016). Alkaloids were present in different parts of female and male *P. longum* plants, as if leaf, root, and spike of female plants and only root of male plants (Prasad *et al.*, 2018).

In order to analyse the transcriptomic profile of black pepper fruit, the raw reads were assembled using the Illumina HiSeq 2000 platform. A number of unigenes and simple sequence repeats (SSRs) were detected from these raw reads. In detail analysis of the fruit transcriptomic study, polymorphic SSRs, lysine/ornithine metabolism-related genes, and housekeeping genes in black pepper were identified,

which provide useful information for further research into piperine synthesis in black pepper (Hu *et al.,* 2015).

Markers such as microsatellites are useful in breeding programs for exploring and characterizing the evolution of plant families. Transcriptomic data could also be used to create a database of expressed genes associated with plant defence and metabolism, which has ecological and biotechnological implications. Using the NGS SOLiD platform, black pepper stem-root tissue was sequenced. The root tissue transcriptome was analysed to obtain de novo assembled transcripts and simple sequence repeats (SSRs) (Gordo *et al.,* 2012). The identified SSRs may have many applications in genetics, biotechnology, and ecology.

Accelerated Extraction of Solvents (ASE) replaces the traditional extraction techniques. For the development of a fast method for sample preparation, ASE was conjunction with Soxhlet extraction. For the first time, piperine and piperlonguminerom were extracted from the root of *P. longum* L using ASE. ASE at 60°C for 20 minutes produced nearly identical results to Soxhlet extraction at 480 minutes. In terms of extraction time and solvent consumption, ASE appears to be an effective alternative to Soxhlet extraction (Rajopadhye *et al.,* 2012). The HPTLC method was discovered to be simple, convenient, and quick for screening and quantification of the active biomarker. Piperine is quantitatively analysed using HPTLC, and six fruits – *Piper nigrum* L., *Piper longum* L., *Piper chaba* Hunt, *Piper cubeba* Hunter and *Piper betle* L are compared. After measuring different mobile phases, toluene-ethyl acetate-diethyl ether 6:4:1 (v/v) produced the best results, with piperine resolution in the presence of other compounds in the sample extracts. The HPTLC plates were scanned at 337 nm, and piperine quantification was performed using a predetermined calibration plot. The amount of piperine was highest in the fruit of *Piper nigrum* L. and lowest in the fruit of *Piper betle* L. (Rajopadhy *et al.,* 2011)

Piper is a taxonomically complex genus due to its wide range of variability and the minute nature of its flowers. Due to high global demand of piper, it is crucial to work on germplasm conservation and further genetic advancement. RAPD is a common genetic marker that is used in population genetics, pedigree analysis, and taxonomic discrimination analysis (Wu *et al* 2006). RAPD analysis of eight distinct Piper species suggests a more distant relationship between *P. betle* and *P. hymenophyllum. P. trichostachyon* and *P. galeatum* were found to be closely related (Sen *et al.,* 2010).

Several endophytic fungi have been found to have the biosynthetic ability to produce the same or similar metabolites encountered in host plants. After the screening various endophytic fungi, *Colletotrichum gloeosporioides* was discovered to have the ability to shape piperine, which was confirmed by HPLC and LCMSS. (Chithra *et al.*, 2014). The presence of mobile piperine was determined by comparing the peaks with standard piperine at comparable retention times. For molecular identification of endophytic fungi with piperine production, BLAST analysis of the ITS area of endophytic fungus PF12 revealed a 100% identity with *Colletotrichum gloeosporioides*.

Inflorescence of *Trachyspermum ammi* is a rich source of secondary metabolites, particularly monoterpenes and thymol. Transcriptomes of four inflorescence tissues from two ajowan ecotypes with varying thymol yields were used to classify putative genes involved in thymol and other monoterpene biosynthesis. *T. ammi* inflorescences RNA was extracted using the TRIzol reagent (Invitrogen, USA). Trinity was used to perform de novo assembly of the resulting pooled clean readings. TransDecoder method is used for classifying Protein Coding Sequence (CDS) regions within all transcript sequences. Protein sequences of predicted ORFs were annotated and functionally analysed by using databases such as KEGG and Uniprot. Metabolic pathways and functional details for each Ajowan unigene, were assigned by using the KEGG (Kyoto Encyclopaedia of Genes and Genome) **(**Howyzeh *et al.*, 2018).

ESTs play an important role in accelerating gene discovery, particularly in non-model organisms where the reference genome sequence is not available, because it is a fast and relatively inexpensive method of analysing the transcribed region of the genome. ESTs are also useful in large-scale expression analysis for improving genome annotation, identifying splice variants, identifying molecular markers, and physical mapping (Rudd, 2003).

In *Glycyrrhiza uralensis* (licorice), a main medicinal ingredient is liquiritin which is a flavonoid and widely used herb in traditional Chinese medicine. The main enzymes and pathways for flavonoid production are explored using gene expression patterns in samples containing varying quantities of flavonoid. Seeds were mutagenized using X-ray irradiation and samples were evaluated by using HPLC analysis. RNA-seq was utilised to analyse gene expression and differentially expressed genes (DEGs) in samples with high flavonoid concentration and one control sample with low flavonoid content. The flavonoid metabolic pathway, terpenoid biosynthetic pathway, plant hormone signal transduction

pathway, plant circadian rhythm pathway, starch and sucrose metabolic pathways were discovered to play essential roles in licorice flavonoid production. Several co-expressed DEGs were validated further using qRT-PCR, indicating that the RNA-Seq findings were accurate and reliable (Hu *et al.,* 2020)

Mangala (2021) evaluated the antimicrobial potential of oleoresin extracted from the wild *P. nigrum* plant. In vitro antimicrobial activity of the oleoresin extracted from the plant's roots and stem has been evaluated by using the Filter paper disc and cut well methods. As the test organisms, five bacteria (*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis, Salmonella typhi and Staphylococcus aureus*) were used along with five fungi (*Aspergillus niger, Aspergillus flavus, Penicillium spp, Drechslera spp* and *Fusarium spp). Bacillus subtilis* was shown to be the most susceptible to oleoresin extract of root and stem among all the bacteria tested, followed by *Staphylococcus aureus, Salmonella typhi, Pseudomonas aerugenosa,* and *E. coli* each with a different inhibitory zone. Penicillium spp. were significantly inhibited by oleoresin extracts from the root and stem, followed by *Fusarium spp., Aspergillus flavus, Aspergillus niger* and *Drechslera spp*. with varied zones of inhibition.

Research has been done on the antimicrobial effects of petroleum ether, chloroform, ethyl acetate and methanol extracts of *Piper chaba* stems and leaves. Different crude extracts were assessed for their antibacterial activities against a number of gram-positive (*Bacillus megaterium, Bacillus subtilis, Sarcina lutea, Staphylococcus aureus, Streptococcus-β-haemoliticus*), gram-negative bacteria (*Shigella sonnei, Pseudomonas aeruginosa, Escherichia coli, Shigella dysenteriae, Shigella shiga, Klebsiella sp. Salmonella typhi*) as well as their antifungal activity against *Aspergillus fumugatous, Mucor sp., Penicillium sp., Human- 3 sp., Aspergillus nigar* and *Havous*. The stem bark extract of *Piper chaba* exhibited mild antibacterial activities against the majority of bacteria tested. All extracts showed no activity against *Staphylococcus aureus*, while no extracts were effective against *Shigella dysenteriosis* Despite being inactive against most organisms, the petroleum ether extract was still active against *Staphylococcus aureus*. Leaf extracts (except petroleum ether) exhibited mild activity against most species of bacteria, but *Shigella dysenteries* was resistant to all extracts. No activity was detected by the extracts against the fungal strains analysed (Roy *et al.,* 2015).

Saraf and Saraf (2014) examined the secondary metabolites and anti-microbial activity of *Piper longum* Linn. Different essential oils, steroids, alkaloids, and arbutin were detected in the various fruit extracts.

Respiratory pathogens i.e., *Staphylococcus aureus, Streptococcus pyogenes* and *Klebsiella pneumoniae* have been used to evaluate the antimicrobial efficacy of the phytochemical extracts of *P. longum. Klebsiella pneumoniae* was found to be the most resistant among the three pathogens of respiratory tract. *Piper longum* extract is effective against both Gram-negative and Gram-positive bacteria, which means the bioactive components present in *Piper longum* fruits such as cardiac glycosides, anthraglycosides, steroids, and essential oil can be used as broad-spectrum antibiotics.

A study by Prasetya *et al.,* (2021) found that the *P. betle* extract exhibited antibacterial activity against two oral pathogenic bacteria *Streptococcus mutans* and *Streptococcus sanguinis* and an oral opportunistic fungal species, *Candida albicans*. *P. betle* extract was tested for antibacterial activity using the well diffusion method and increasing concentrations of *P. betle* extract from 0.5% to 1% inhibited the growth of S. mutans, *S. sanguinis* and *C. albicans* with increasing diameters of inhibition detected. When compared to *S. sanguinis* and *C. albicans*, *S. mutans* was the most susceptible to *P. betle* extract. Different cell types are most likely to be responsible for the differences in sensitivity between these species.

A known alkaloids Chabamide I, Piperine and new alkaloids Chingchengenamide A were isolated from the methanol extract of dichloromethane fraction of *Piper chaba* leaves for determine the in vitro antimicrobial activity of these compounds. The total ethyl acetate extract and pure compound Chabamide I, Piperine and Chingchengenamide A of *P. chaba* H. were dissolved in sufficient amount of solvents and evaluated by agar diffusion method at a dose of 300 µg/disc. *Candida albicans, Aspergillus niger,* and *Sacharomyces cerevacae*; gram positive bacteria including *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Sarcina Lutea,* and *Staphylococcus aureus* while gram negative bacteria including *Escherichia coli, Salmonella paratyphi, Salmonella typhi, Shigella boydii, Shigella dysenteriae,* and *Vibrio mimicus* were tested. The diameter of the zone of inhibition was determined using Kanamycin (30µg/disc) discs as standard antifungal and antibacterial agents. Chabamide showed good antimicrobial efficacy against all of the microorganisms, fungi, and standard Kanamycin used in the study. (Shandhi *et al.,* 2020).

Rahman and Al-Reza (2011) study efficacy of the essential oil and different leaf extracts of *Piper chaba* Hunter for suppressing the growth of some important phytopathogenic fungi. The plant-pathogenic fungi

used in this study were *Fusarium oxysporum, Phytophthora capsici, Colletotrichum capsici, Fusarium solani, Rhizoctonia solani, Sclerotinia sclerotiorum* and *Botrytis cinerea*. Cultures of each of these fungal species were kept on potato-dextrose agar (PDA) slants and stored at 4˚C. Except for *B. cinerea* and *S. sclerotiorum*, the essential oil and organic extract of *P. chaba* Hunter had a significant antifungal action as a growth inhibition against all these fungi tested in the range of 55.1–70.3% and a minimum inhibitory concentration ranging from 125 to 500µg/ml. The presence of significant components e.g., a-humulene, caryophyllene oxide, viridiflorol, globulol, b-selinene, spathulenol, (E)-nerolidol, linalool, 3-pentanol, and p-cymene and minor components in the oil could be attributed to antimicrobial activity.

PROPOSED METHODOLOGY

1. **Establishment of *P. chaba* plants in DEI botanical garden**.

- *Piper chaba* will be procured from NBPGR (National Bureau of Plant Genetic Resources), Thrissur, Kerala and established in the botanical garden of DEI.
- Soil and water analysis will be done, and nutrients dose will be given according to plant requirement.

2. Quantification of secondary metabolites in different parts of the plant

- TLC (Biradar *et al.,* 2013) and HPLC (Kamal *et al.,* 2016) methods will be used.

3. Analysis of anti-microbial activity of secondary metabolites

- Agar well diffusion and Disc diffusion method (Khan *et al.,* 2013) will be performed.

4. Transcriptomic analysis of different parts of the plant

- Next generation sequencing will be performed.

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